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Published in:
Clinical and Experimental Allergy

DOI:
10.1111/cea.12972

Publication date:
2017

Document Version
Peer reviewed version

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Accepted author manuscript

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Running title: Tiotropium and airway hyperresponsiveness

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Funding: Funded by a departmental unrestricted educational grant

Conflicts of Interest: Dr. Jabbal reports personal fees and non-financial support from Chiesi Pharma, personal fees and non-financial support from Pfizer, personal fees and non-financial from Astra Zeneca, non-financial support and other from Napp, outside the submitted work. Dr. Manoharan has nothing to disclose. Dr. Lipworth reports personal fees and other from Boehringer Ingelheim, grants and personal fees from Chiesi , personal fees from Dr Reddys , personal fees from Cipla , grants and personal fees from Teva, grants and personal fees from Meda , personal fees from Sandoz, during the conduct of the study; grants from Astra Zeneca , grants from Janssen , grants from Roche , outside the submitted work.

Word Count (abstract): 241
Word Count (text): 2770

Abstract:

Background: Tiotropium is a long acting antimuscarinic (LAMA), licenced as triple therapy with inhaled corticosteroid and long acting beta-agonist (ICS/LABA). There may be a synergistic benefit between LAMA and LABA as a consequence of receptor cross-talk, which in turn could modify beta-2 receptor down-regulation and associated tolerance induced by LABA.

Objective: We hypothesise this mechanism may result in a reduction of airway hyperresponsiveness (AHR) when using triple therapy.

Methods: We evaluated 14 non-smoking asthmatics using an open-label, randomized crossover design. ICS with Indacaterol and Tiotropium (IND/TIO) vs ICS with Indacaterol (IND) over 4 weeks with challenge performed after 1st and last doses at trough.

Results: We found no significant difference in mannitol sensitivity, expressed as the provocative dose of mannitol required to reach a 15% drop in FEV_1, or mannitol reactivity, expressed as the response dose ratio (RDR: max % fall in FEV1 / cumulative dose) , when comparing ICS/IND/TIO to ICS/IND. Geometric mean fold differences for RDR comparing single and chronic dosing were 3.26 fold (95%CI 1.46-7.29) and 2.51 fold (95%CI 1.32-4.79) for IND and IND/TIO respectively. Furthermore, salbutamol recovery post challenge was significantly blunted after chronic compared to single dosing with either IND ($P<0.005$) or IND/TIO ($P<0.05$).

Conclusion & Clinical Relevance: Our data suggests that concomitant tiotropium does not modify the bronchoprotective tolerance induced by Indacaterol, in turn suggesting that cross-talk may not be clinically relevant when using triple therapy. This study was registered on clinicaltrials.gov as NCT02039011.

Abbreviations:

AHR: Airway hyperresponsiveness

ACQ: Asthma control questionnaire

AX: Reactance area under the curve

FeNO: Exhaled nitric oxide

ICS: Inhaled corticosteroid

IND: Indacaterol

IOS: Impulse oscillometry

LABA: Long acting beta-2 agonist

LAMA: Long acting muscarinic antagonist

PD_{15}: Provocative dose of mannitol required to reduce FEV_1 by 15%

RDR: Response dose ratio

TIO: Tiotropium

Introduction

Tiotropium (TIO) is a long acting muscarinic antagonist (LAMA), which is functionally selective for the post junctional M3 muscarinic receptor, found on airway smooth muscle [1]. TIO reduces asthma exacerbations by 21% in patients when used as add-on therapy in patients receiving inhaled corticosteroids and long-acting beta-agonists (ICS/LABA)[2]. Whilst blocking the M3 receptor inhibits acetylcholine induced bronchoconstriction, TIO exhibits only modest improvements in FEV_1, which amounts to approximately 100ml at trough [2, 3] ,which is less than the minimally important difference of 230ml [4]. It is therefore hard to explain the protective effect on exacerbations on solely the basis of this small improvement in airway calibre alone [5].

One mechanism by which TIO may exhibit its protective effects is by attenuating airway hyperresponsiveness (AHR), via blockade of the post junctional M3 muscarinic receptor, resulting in reduced response to cholinergic transmission [6]. M3, however, is not the only muscarinic receptor to contribute to increased airway tone and AHR; asthma is also associated with impaired pre-junctional M2 function [7] [8]. The pre-junctional M2 is an autoreceptor, as it is stimulated by acetylcholine to reduce further acetylcholine secretion. In asthma, the loss of this negative feedback mechanism results in increased AHR. Moreover, it has been postulated that both pre-junctional beta-2 and M2 receptors are inhibitory to the release of acetylcholine and that there is crosstalk between these receptor types [7, 8]. Hence it might be expected that chronic dosing with LABA might remove the brake to acetylcholine release as a consequence of down-regulation and subsensitivity of pre-junctional inhibitory beta-2 receptors, resulting in augmented cholinergic transmission and bronchoconstriction [7]. In this regard, TIO rapidly dissociates from M2 receptors, unlike its affinity for post junctional M3 receptors, thereby facilitating additional inhibition of M2 receptors and reduced pre-junctional acetylcholine release. This functional M3 selectivity may be a possible mechanism by which it reduces exacerbations in asthma by attenuating AHR [1].

Another possible mechanism is that muscarinic M3 receptors promote beta-receptor desensitization through protein kinase C–mediated phosphorylation [9], hence inhibition of this effect by TIO may protect the beta-2 receptor from acetylcholine induced heterologous desensitization by acetylcholine[10]. In this regard looking at the converse situation, tiotropium has been shown to protect against propranolol induced bronchoconstriction [11].

TIO may also reduce exacerbations via a putative anti-inflammatory action by inhibiting the paracrine effects of acetylcholine on inflammatory cells [12] . TIO has been shown to exhibit inhibitory effects on the development of airway remodelling in the animal model of antigen induced asthma[12, 13] . In vitro data have also suggested that there may be an anti-inflammatory synergy between LABA and LAMA, via the cAMP pathway [9].

Pointedly no studies have looked at effects of TIO on AHR assessed by bronchial challenge using non cholinergic agents. One study showed that as expected TIO produced prolonged functional antagonism of M3 mediated smooth muscle constriction induced by the cholinergic agonist methacholine [14]. As TIO is only currently indicated as add-on therapy to ICS/LABA [15], the objective of this study was to evaluate the impact of adding TIO to ICS/LABA on AHR, in patients with persistent asthma and whether TIO might also prevent against LABA induced subsensitivity[7]. We chose an indirect bronchial challenge, namely mannitol, as this is thought to more closely reflect physiological stimuli and acts by release of pro-inflammatory mediators [16]. Moreover mannitol challenge has been shown to be related to an inflammatory phenotype in asthma [17-19].

Patients and Methods:

Non-smoking male or female patients, aged at least 18 years, with persistent asthma already receiving ICS or ICS/LABA attended for a screening visit. Participants were recruited from the National Health Service (NHS Scotland) boards of Tayside and Fife, and also our existing database of asthma patients, at the Scottish Centre for Respiratory Research, in Ninewells Hospital & Medical School, Dundee, Scotland. Participants had to have a minimum FEV_1 of >50% predicted and be mannitol responsive i.e. provocative dose required to reduce FEV_1 by 15% (PD15) <635mg, to be enrolled. Participants were also required to have no history of respiratory tract infection or oral corticosteroid use, in the last three months prior to screening. After initial screening, any LABA therapy was first withdrawn for 2 weeks followed by halving the ICS dose, to a minimum of 400µg/day (as beclometasone equivalent dose). If patients were on secondary controllers such as leukotriene receptor antagonists, these were also stopped. Participants then entered a 2 week run in on this dose of ICS, which was then continued throughout the study.

The trial was a single centre, randomised open label cross-over design. Patients received either 4 weeks of indacaterol (Onbrez Breezhaler, Novartis, Calberley ,UK) alone at a dose of 150µg OD (IND), or combined with tiotropium (Spiriva Handihaler, Boerhinger Ingelheim, Bracknell ,UK) 18µg OD (IND/TIO) as add-on to pre-existing ICS. There was a 2 week washout in between treatments while continuing to take the same dose of ICS. This washout was sufficient to minimise the possibility of carry-over effects of both IND and TIO [20].

Including screening, there were 7 visits in total (figure 1.). Visits were performed, in the mornings, at baseline after run-in and washout, and at 24 hours (i.e. trough) after the first and last doses of each randomised treatment period. Patients were allowed short acting beta-2 agonists (SABAs) as a reliever during the study, but were asked to abstain from SABA use at least 6 hours before each visit. The visits were conducted in the mornings (8am-10am). Participants were asked to record study medication use on a diary, and compliance was checked with returned empty capsule counts. This study was registered on clinicaltrials.gov as NCT02039011. The study was approved the Tayside committee for medical ethics (reference: 13/ES/0072) and full informed consent was obtained from all patients.

The primary outcome was mannitol challenge. This was performed as previously described[21] using a dry powder inhaler (Aridol Pharmaxis Ltd, Sydney, Australia) and increments up to a maximum cumulative dose of 635 mg. Mannitol sensitivity was expressed as the provocative dose of mannitol required to reach PD15, this was calculated by interpolation of the log-linear dose–response curve. The data for PD15 were log transformed before analysis. Mannitol reactivity was expressed as the response dose ratio (RDR) –i.e. maximum % fall in FEV_1 divided by the final mannitol dose. Impulse oscillometry, a secondary outcome, (Jaeger Masterscreen IOS, Hochberg, Germany) was performed as previously described [22] in triplicate, whilst subjects wore a nose clip, sealed lips tightly, and breathed quietly for 30 seconds, in accordance with manufacturer's guidelines. Resistance at 5 Hz (R5) and 20Hz (R20) is a measure of total and central airway resistance respectively, hence peripheral airway resistance was ascertained by the difference between R5 and R20. Lung compliance as its reciprocal reactance (X) and the area under the reactance curve (AX) was also measured. A SuperSpiro spirometer (Micro Medical Ltd, Chatham, Kent, United Kingdom) was used to perform spirometry in triplicate in accordance with European Respiratory Society guidelines[23]. After mannitol challenge, salbutamol (400µg) was administered and 30 minute recovery recorded. Exhaled nitric oxide (FeNO) was performed using an NIOX MINO analyser (Aerocrine AB, Solna, Sweden), in accordance with the published guidelines [24]. Asthma control questionnaire (ACQ-7) was measured using the standard 7 point paper

questionnaire[25] (Qoltech, UK) . Randomisation was done with a computer generated code held by the Clinical Trials Pharmacy.

Data Analysis

The study was powered at 80% to detect a minimal important difference of one doubling dose in mannitol PD 15 (the primary outcome), as change from baseline, comparing indacaterol alone with indacaterol plus tiotropium, after single and chronic dosing, and a within-subject SD of 1.3 doubling dose, requiring a sample size of 14 using a crossover design, with alpha error of 0.05 (2 tailed). All data were first examined for normality and distribution. Repeated measures analysis of variance (ANOVA) was carried out assessing for treatment and sequence effects for the cross-over design. Where overall significance was found on ANOVA, Bonferroni corrected pairwise comparisons were then carried out. Thus, pairwise comparisons are reported as either significant ($p<0.05$, two-tailed) or not. Statistical Analysis was done using IBM SPSS (version 22, IBM analytics, New York).

Results:

The participant flow for the trial is shown in the consort diagram (Figure 2), of the 39 patients screened 18 were randomised and 14 completed per protocol. Of the 14 ICS treated asthmatic patients analysed, 12 had at least one positive skin prick test to common aeroallergens, mean age was 46 years , mean FEV_1 86% predicted , mean BMI 30kg/m^2, mean R5 160% predicted , and mean ICS dose 693µg/day (beclometasone equivalent dose). No patients were current smokers, two were ex-smokers with a mean pack year history of 2.6. Values comparing mean ICS dose pre and post step down were 693 vs 429 µg/day ($P< 0.05$).

Data for all outcomes according to study visits are summarised in Table 1. All outcome measures at first baseline and second baseline were assessed for carryover effect in order of sequence. There was no statistical difference between baseline data justifying the use of a pooled baseline value for comparison with randomised treatment arms. This confirmed an adequate washout period. In particular, there was no significant difference between mean baseline values for the primary outcome of mannitol PD15: 383mg vs 387 mg.

There were significant improvements ($P<0.05$) in mannitol PD15 and RDR with IND or IND/TIO vs baseline after single but not chronic dosing (Figure 3). There was a significant difference ($P<0.05$) in RDR between single and chronic dosing for both treatments: geometric mean fold differences were 3.26 fold (95%CI 1.46-7.29) and 2.51 fold (95%CI 1.32-4.79) for IND and IND/TIO respectively. Furthermore, salbutamol recovery post challenge was significantly blunted after chronic compared to single dosing with either IND ($P<0.005$) or IND/TIO ($P<0.05$) (Figure 4 and table 1).

IOS measures including R5, R5-R20, and AX were all significantly improved ($P<0.05$) with both treatments compared to baseline after single and chronic dosing. FEV_1 and FEF_{25-75} were significantly better after single dosing with both treatments ($P<0.05$) but only after chronic dosing with IND/TIO ($P<0.05$). There were no significant differences between treatments after chronic dosing for either mannitol AHR, spirometry or IOS outcomes. FeNO was unchanged with either treatment compared to baseline. ACQ was also unchanged by either treatment.

Discussion:

Our results showed improvements in mannitol AHR with both treatments after single dosing which were not maintained after repeated exposure, in addition to blunting of salbutamol recovery. This is likely to be indicative of agonist induced down-regulation and uncoupling of beta-2 receptors and associated tolerance of response .The loss of bronchoprotection induced

by indacaterol and associated cross tolerance seen as blunted salbutamol recovery has previously been well documented with other twice daily LABA's in patients taking concomitant ICS [26-28] [29] [30]. Indacaterol has a high degree of intrinsic efficacy at the beta-2 receptor being 73% compared to the effect of isoprenaline in vitro.[31] In another study using isolated human bronchi the maximal relaxant response was 77% for indacaterol versus 94% for formoterol [32]. In this regard prolonged stimulation with a high efficacy agonist like indacaterol would be expected to result in marked down regulation and uncoupling of beta-2 receptors as has been previously shown with formoterol [28, 33-35].The loss of bronchoprotection was seen with indacaterol at 24 hours after the last dose at trough , when the airway might be particularly vulnerable to exogenous constrictor stimuli immediately prior to the next dose. The degree of bronchoprotection loss was the same with both treatments while chronic treatment with IND/TIO was no different compared to IND alone. Hence it can be concluded that we did not see any clinical evidence of crosstalk between muscarinic and beta-2 receptors ,at least in terms of bronchoprotective subsensitivity using indirect challenge with mannitol [7]. The absence of any bronchoprotection seen with TIO is consistent with similar findings with ipratropium using direct acting histamine challenge [36] . In terms of the choice of challenge agent, mannitol was chosen as it is a well validated [37] indirect challenge and hence better reflects other physiological stimuli than direct challenges such as methacholine or histamine. Furthermore at the time of doing the study adenosine 5' monophosphate (AMP) for human use was not commercially available. Whilst it is noted that response to mannitol is influenced by ICS [38], our patients had to be mannitol responsive at the first visit whilst taking a stable ICS dose, which remained constant throughout the study. Therefore we felt that any changes in mannitol AHR would only reflect the impact of bronchodilator treatments. Furthermore the PD_{15} and RDR values were not statistically different between first and second baseline, suggesting no carryover effects between randomised treatment arms.

For IOS and spirometry, both treatments conferred improvements which were maintained after chronic dosing. As was the case with AHR, we found no significant differences in pulmonary function outcomes after chronic dosing comparing between IND/TIO and IND alone. Previous studies in more severe patients have shown that TIO in addition to ICS/LABA results in approximately 100ml improvement in FEV_1 [2], in turn suggesting that improved airway calibre per se is unlikely to be the explanation for reduced exacerbations[5] . We had originally considered that IOS might be more sensitive than spirometry at picking up subtle differences between double and triple therapy for bronchodilator effects measured at trough [39] [40].In the presence of a raised baseline R5 value of 160 % predicted, one might expect there to plenty of room for further improvement comparing double and triple therapy, which was not the case. Further studies are indicated to look at whether IOS is more sensitive to effects of TIO in more severe patients.

There was no improvement in ACQ score, this reflecting the mean baseline value of 0.72 which is less than the 0.75 cut off value for optimal control[41] . However, the failure of add-on therapy with LAMA to improve ACQ scores was also seen by Peters et al in a much larger and more severe cohort [42]. FeNO was unchanged with either treatment, which could be explained by levels being already suppressed by concomitant ICS. Nonetheless, one would still expect the addition of TIO to have contributed to a modest further reduction from a mean baseline value of 30ppb, as shown in another study in more severe patients looking at triple therapy [43].

The clinical relevance of our data is that when using triple therapy, at least in asthma, any effects of LAMA on exacerbations is unlikely to be due to bronchoprotective effects. Moreover concomitant LAMA does not mitigate tolerance induced by LABA or cross tolerance to salbutamol. Hence for patients taking ICS/LABA who might experience reduced protection against bronchoconstrictor stimuli, adding in a LAMA will not alleviate the situation, although it might conceivably still produce fewer exacerbations. The caveat is that our patients only had mild to moderate asthma and hence we did not see any significant additive bronchodilator

effects with LAMA. In other words if LAMA had produced altered airway geometry then perhaps we might have seen some additional bronchoprotection. Against this is the previous observation of Britton et al where ipratropium did produce a dose related bronchodilator response which was disconnected from any effects on AHR to histamine challenge [36].

We accept that our study has limitations in that our patients were initially well controlled Moreover, our sample size was not powered to detect additional bronchodilation with TIO. As airway, geometry is an important determinant of bronchoprotection our negative findings with TIO on mannitol challenge might simply reflect the lack of additional bronchodilator effect with TIO. Although we did not have a comparator limb with TIO alone, one would have expected to see additive effects on AHR after chronic dosing when the bronchoprotective effect of LABA had diminished, in terms of there being room for potential further improvement after the last dose. One could always argue that TIO is only indicated for use as add-on to ICS/LABA, as was the case in the present study, and hence performing a study looking at TIO alone or in conjunction with ICS would have no clinical resonance. Finally, we acknowledge that we did not measure either sputum or blood eosinophils in the present study, although in that respect our patients were selected a priori on the basis of AHR.

In conclusion, TIO did not modify the bronchoprotective tolerance induced by indacaterol or the cross tolerance seen on blunting of salbutamol recovery. Further studies perhaps involving bronchial biopsy might provide an insight into the putative anti-inflammatory action of TIO in asthma to help further elucidate the mechanism by which it reduces exacerbations in patients taking ICS/LABA.

Acknowledgements: Mannitol was provided by an unrestricted educational grant from Pharmaxis, Sydney Australia. We would like to acknowledge the patients who volunteered to take part in our trial and Ms Ashley Morrison for helping to coordinate the study visits.

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548 **Table 1.**

		INDACATEROL		INDACATEROL + TIOTROPIUM	
	Pooled baseline	**Single dosing**	**Chronic dosing**	**Single dosing**	**Chronic dosing**
FEV_1 (L)	2.56 (2.18-2.95)	2.69 (2.28-3.10)*	2.64 (2.26-3.02)	2.78 (2.38-3.19)*	2.71 (2.33-3.09)*
FEV1 percent predicted (%)	87 (78-97)	91 (82-100)*	90 (81-100)	95 (85-105)*	93 (83-102)*
FEF_{25-75} (L)	1.79 (1.22-2.36)	2.02 (1.39-2.65)*	1.91 (1.25-2.57)	2.22 (1.49-2.95)*	1.94 (1.38-2.49)*
R5 (kPa/l.s)	0.54 (0.44-0.64)	0.45 (0.37-0.52)*	0.44 (0.37-0.50)*	0.39 (0.34-0.43)*	0.45(0.39-0.50)*
R5-R20 (kPa/l.s)	0.14 (0.07-0.22)	0.07 (0.03-0.11)*	0.07 (0.04-0.10)*	0.05 (0.03-0.07)*	0.08 (0.04-0.11)*
AX (kPa/l)	1.63 (0.58-2.68)	0.76 (0.34-1.19)*	0.68 (0.43-0.92)*	0.44 (0.25-0.63)*	0.78 (0.48-1.09)*
RDR (%/mg)	0.037 (0.025-0.055)	0.011 (0.005-0.026)*	0.037 (0.023-0.061)†	0.015 (0.008-0.029)*	0.035 (0.018-0.070)†
PD_{15} (mg)	390 (291-521)	537 (438-619)*	455 (342-606)	487 (329-624)*	388 (255-593)
FENO (ppb)	30 (20-45)	30 (20-44)	30 (20-45)	32 (23-45)	29 (19-44)
Salbutamol Recovery (%.min)	47 (-79 - 172)	33 (-47 – 113)	259 (196 – 322)*	77 (19-136)	239 (177-300)*
ACQ7	0.72 (0.48-0.95)		0.44 (0.24-0.63)		0.50 (0.27-0.73)

Values are presented as mean (95% CI)
*Denotes significant (P<0.05) difference from pooled baseline.
†Denotes significant difference (P<0.05) between single and chronic dosing within treatment groups.
No statistically significant differences observed between Indacaterol vs Indacaterol + Tiotropium when comparing single vs chronic dosing at trough. Salbutamol recovery is expressed as the area under the curve (AUC) for 30 minutes.

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Figure Legends

Figure 1.
Flowchart.

Figure 2.
Consort diagram.

Figure 3.
Effects of randomized treatments (as add on to ICS) compared to baseline on (a) mannitol sensitivity and (b) reactivity. P value denotes significant difference for randomised treatments compared to baseline. There was also a significant difference between single and chronic dosing for reactivity with both treatments. There were no differences between treatments. Values are geometric means and 95% CI.

Figure 4.
Effects of single and chronic dosing with either (a) indacaterol alone or (b) indacaterol +tiotropium (as add on to ICS) on salbutamol (400ug) recovery post challenge. P value denotes significant overall blunting of the salbutamol recovery comparing chronic vs single dosing. Values are means and SEM.